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The Comparative Cell Cycle and Metabolic Effects of Chemical Treatments on Root Tip Meristems. III. Chlorsulfuron

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Abstract. Intact and excised cultured pea roots (*Pisum sativum* L. cv Alaska) were treated with chlorsulfuron at concentrations ranging from 2.8 $\times$ 10^{-4} M to 2.8 $\times$ 10^{-6} M. At all concentrations this chemical was demonstrated to inhibit the progression of cells from G_2 to mitosis (M) and secondarily from G_1 to DNA synthesis (S). The S and M phases were not directly affected, but the transition steps into those phases were inhibited. Total protein synthesis was unaffected by treatment of intact roots with 2.8 $\times$ 10^{-6} M chlorsulfuron. RNA synthesis was inhibited by 43% over a 24-h treatment period. It is hypothesized that chlorsulfuron inhibits cell cycle progression by blocking the G_2 and G_1 transition points through inhibition of cell cycle specific RNA synthesis.

All cells in the plant body are driven by the interactive yet independent metabolic activity of two cycles, the cell cycle and the growth cycle (Rost 1977). The cell cycle, consisting of the stages G_1, S, G_2, and M is a sequential progession of steps whereby cells are metabolically prepared for and pass through DNA synthesis (S) and mitosis (M). This sequence requires protein synthesis and nucleic acid synthesis to insure progression.

The growth cycle consists of general metabolic events, such as respiration, plus more specific events, such as those required for cell enlargement and cell differentiation. Operation of both cycles is dependent upon continuous metabolic activity, yet their separation is witnessed by cell behavior after certain stress treatments. Treatment of meristems with drugs like colchicine will arrest cell cycle progression by direct mitotic interference without a concomitant

inhibition of cell enlargement and cell differentiation. Evans (1965) observed a similar stress behavior after X-irradiation inhibition of mitotic progression followed by premature differentiation of tissues within the meristem.

Ray (1980, 1982) has reported on the mode of action of a new herbicide, chlorsulfuron (2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)aminocarbonyl]benzenesulfonamide). This chemical, intended for control of broad leaf weeds in cereal crops, showed metabolically interesting responses in several experimental systems. Chlorsulfuron (2.8×10^{-5} M) did not inhibit indoleacetic acid-induced elongation of subapical etiolated pea stems, cytokinin-induced cell expansion of cucumber cotyledons, nor gibberellic acid-induced elongation of lettuce hypocotyls (Ray 1982). However, at a 10 times lower concentration chlorsulfuron did induce an 87% reduction in mitotic index of *Vicia faba* L. root tips after an undisclosed treatment duration. This chemical, likewise, did not inhibit O_2 uptake in pea root tips, O_2 evolution in isolated pea chloroplasts, or CO_2 fixation in spinach cells, but it did inhibit ^{3}H-thymidine incorporation into DNA of corn root tips by 60% in 2 h without a reduction in its uptake.

The results of Ray's experiments suggest chlorsulfuron as a potentially useful experimental chemical capable of application as a metabolic probe to separate the cell cycle from the growth cycle. In the research reported here, a series of experiments are discussed that were designed to examine the effect of chlorsulfuron on cell cycle progression.

Materials and Methods

Pea seeds, *Pisum sativum* L. cv. Alaska, were germinated aseptically in vermiculite. After 3 to 5 d, seedlings were suspended in 1/4x aerated Hoagland's solution for a 12-h equilibration period. Seedlings were then transferred to fresh Hoagland's solution (Wilt and Wessells 1967) with or without chlorsulfuron at concentrations ranging from 2.8×10^{-6} M (1 ppm) to 2.8×10^{-4} M (100 ppm), with or without ^{3}H-thymidine (specific activity 6 mCi/mm) at 0.1 μCi/ml. Five roots were collected per sample at various times after treatments were started. Root tips were hydrolyzed in 5N HCl at room temperature, stained in Schiff's reagent by the Feulgen method (Gomori 1952), and squashed onto microscope slides. In isotope autoradiography studies, slides were coated with Kodak NTB3 liquid emulsion, exposed in a refrigerator for 5–7 d in the dark, developed, and scored for labeled and unlabeled division figures and interphase cells. A minimum of 4,000 cells were scored for each data point. Standard error of the means is shown on each graph as appropriate. All experiments were repeated at least two times. A Zeiss MPMO3 scanning photometer coupled to an ISC 3650 Zonax computer was used for microspectrophotometric studies. An internal standard of measured prophase cells was used to determine the range of DNA content in a G_2 nucleus.

Uptake and incorporation of isotopically tagged thymidine, uridine, and reconstituted protein hydrolysate was measured by scintillation counting to determine the short-term effects of chlorsulfuron on DNA, RNA, and protein synthesis. Isotopes (Schwarz/Mann) at the following concentrations were used: ^{3}H-thymidine (0.1 μCi/ml, sp. act. 6 mCi/mm); ^{3}H-uridine (0.5 μCi/ml, sp. act.

25 Ci/mm); ^{3}H-reconstituted protein hydrolysate (0.5 μCi/ml, sp. act. unknown). Roots were sampled at 20 min, 40 min, 1, 4, 8, 12, and 24 h with and without chlorsulfuron. Immediately after sampling, 5–10 roots were placed in the isotope solution for 30 min. After labeling, the 2-mm tips were excised and placed into cold 80% ethanol. Tips were macerated in a glass tissue grinder and poured onto a GF/A Whatman filter on a vacuum filter apparatus. The filter was washed with 80% ethanol, air dried, placed into Beckman Ready-Sol EP scintillation fluid, and counted in a Beckman LS9800 scintillation counter. These counts were plotted as incorporation per root. Uptake was measured by placing 1 ml of the filtrate solution in the scintillation fluid, counting it, and multiplying by a factor to account for the total volume of filtrate and then dividing by the number of roots sampled.

As a reference, the cell cycle duration of pea root meristem cells is 14 h; with G_1, S, G_2, and M being 5 h, 4.5 h, 3 h, and 1.3 h, respectively (Van't Hof 1974).

Results

The G_1 to S Response

The percentage of labeled interphase cells after continuous treatment with chlorsulfuron, at three concentrations, is shown in Fig. 1. The control shows a growth fraction of approximately 88%. This is an estimate of the proportion of the meristem that is actively cycling. During the first 4 h all treatment concentrations follow approximately the same curve. This indicates that the progression of cells into DNA synthesis from G_1 is not immediately affected by the inhibitor during this treatment period. It could also mean that uptake of the chemical is not rapid enough to inhibit S. After 4 h the 2.8×10^{-5} M (10 ppm) and 2.8×10^{-4} M (100 ppm) treatments have deviated from the control. This reduced number of labeled interphase cells indicates that cell progression from G_2 and perhaps G_1 into DNA synthesis must have been affected by chlorsulfuron at these concentrations. There is no further increase in labeled interphase cells at these concentrations suggesting that no further cycling can occur. At 2.8×10^{-6} M (1 ppm), the deviation from the control does not occur until after 8 h of treatment, corresponding to a G_2 inhibition response.

The inset at the top of the graph shows the total labeled interphase cells as a percentage of control. After 2.8×10^{-6} M treatment over a 24 h period, 95.6% of the cells were labeled compared to the control. In the 2.8×10^{-5} M and 2.8×10^{-4} M treatments, 82.6% and 71.6% of the control were labeled.

Fig. 2 shows the effect of 2.8×10^{-6} M chlorsulfuron on S progression as measured by ^{3}H-thymidine incorporation. Uptake was unaffected by 2.8×10^{-6} M, but incorporation was inhibited before 4 h. Little DNA synthesis occurred after that time as indicated by the very slight increasing slope in the incorporation curve after 4 h. This is an indication that even at the lowest concentration chlorsulfuron may inhibit G_1 entry into S.

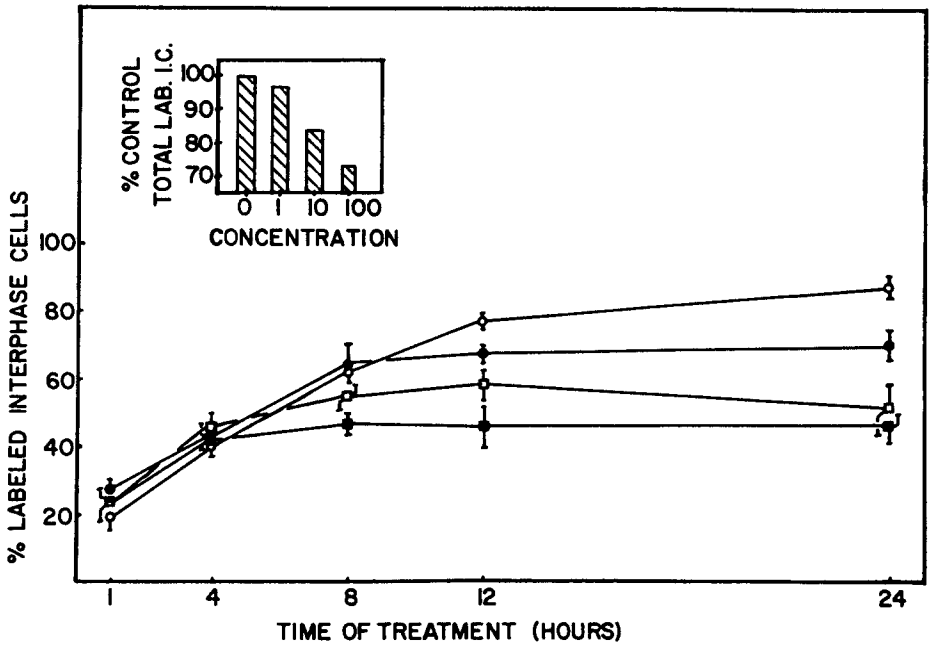

Fig. 1. Percentage of interphase cells in the control (open circles), 2.8×10^{-6} M (closed circles), 2.8×10^{-5} M (open squares), and 2.8×10^{-4} M (closed squares) chlorsulfuron treatment. Inset shows the number of cells able to transit G_1 into S after 24 h of chlorsulfuron treatment.

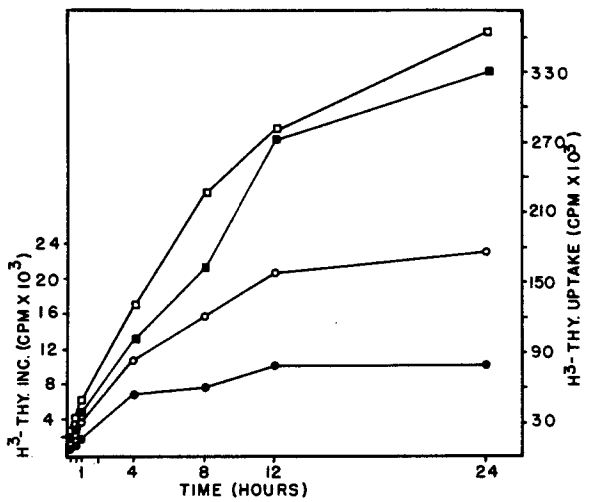

Fig. 2. ^{3}H-thymidine incorporation and uptake, 2.8×10^{-6} M chlorsulfuron (closed symbols) and control (open symbols). The circles represent incorporation, and the squares uptake.

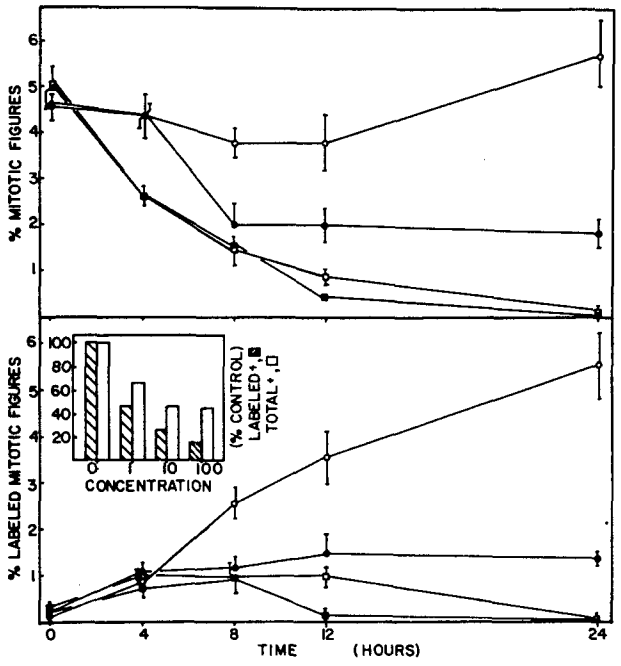

Fig. 3. Progression of cells through mitosis and from G_2 to mitosis. Top panel shows a control (open circles), and 2.8 $\times$ 10^{-6} M (closed circles), 2.8 $\times$ 10^{-5} M (open squares), and 2.8 $\times$ 10^{-4} M (closed squares) treatments on the percentage of mitotic figures present during 24 h of treatment. The lower panel represents the progression of cells previously labeled in S as they progress into G_2 and appear as labeled mitotic figures.

Mitotic Entry Response

The top panel in Fig. 3 shows the percent mitotic figure curves for the 0 to 2.8 $\times$ 10^{-4} M treatments. The gradual mitotic entry inhibition response for all concentrations indicates that chlorsulfuron is inhibiting the entry of G_2 cells into M. The mitotic apparatus itself was unaffected, since no aberrant mitotic figures were observed and there was no change in mitotic stage distribution even when the numbers of mitotic figures were reduce (data not shown). This observation is in agreement with Ray (1980).

The bottom panel of Fig. 3 shows the percent labeled mitotic figures with different treatments. These curves are composed of those cells previously labeled in S, which have progressed through G_2 and into M as labeled division figures. During the first 4 h there was a small parallel increase in the number of labeled division figures in the control and all chlorosulfuron concentrations. In the controls the number of labeled mitotic figures gradually increased until all were labeled. In all three chlorsulfuron concentrations there is no further increase in the number of labeled mitotic figures after 4 h. This shows that cells are not capable of passing through G_2 and going into mitosis. This experiment provides direct evidence that chlorsulfuron inhibits the transit of cells through G_2.

The inset shows the total number of division figures for the three treatments as a percentage of control—67%, 46%, and 45% for 2.8 $\times$ 10^{-6} M, 2.8 $\times$ 10^{-5} M, and 2.8 $\times$ 10^{-4} M, respectively. The total number of labeled division figures was 45.8% (2.8 $\times$ 10^{-6} M), 25.6% (2.8 $\times$ 10^{-5} M), and 18.6% (2.8 $\times$ 10^{-4} M). The greater severity in the percentage contribution of labeled division

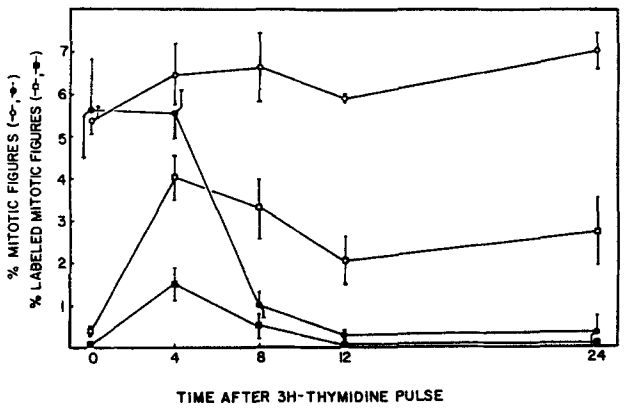

Fig. 4. One h exposure to ^{3}H-thymidine in controls (open symbols) and 2.8×10^{-6} M chlorsulfuron (closed symbols).

figures is an indication of the strength of the G_2 inhibition effect as the concentration was increased.

Pulse Label Experiment

In this experiment, seedlings were placed in Hoagland's solution containing chlorsulfuron at 2.8×10^{-6} M concentration (Fig. 4). Before being placed in the inhibitor, all roots were exposed to ^{3}H-thymidine for 1 h. As the cells pass through G_2 and mitosis after being labeled, the mitotic figures should be labeled and then should disappear as they pass through mitosis. At 4 h the percentage of labeled mitotic figures reaches its maximum point; after that it decreases and remains constant. This indicates that the labeled mitotic figures travel through the system approximately 4 h after the labeling time. In the chlorsulfuron treatment the percent mitotic figures remain approximately constant for the first 4 h and then decrease rapidly, indicating a G_2 inhibition. After that, the number of mitotic figures remains quite low. The peak of the maximum percent labeled mitotic figures corresponds timewise to the peak of the control. This indicates that chlorsulfuron does not affect the timing of cell progression through the cell cycle. However, there is a decrease in the percentage of labeled mitotic figures. This reinforces the earlier conclusion that chlorsulfuron has a strong inhibitory effect on the progression of cells through G_2.

Composite Graph

Fig. 5 shows the results of a composite experiment designed to demonstrate the total effect of 2.8×10^{-6} M chlorsulfuron. The slope of the percent labeled interphase cells curve (0.5 cells/h) for the control indicates the relative rate of cell progression from G_1 into DNA synthesis. After 24 h the growth fraction, or total number of cycling cells, exceeds 80%. The number of mitotic figures remained constant at just under 4%, and the progression of previously labeled cells into mitosis increased gradually to show the progression of cells from G_2 into mitosis.

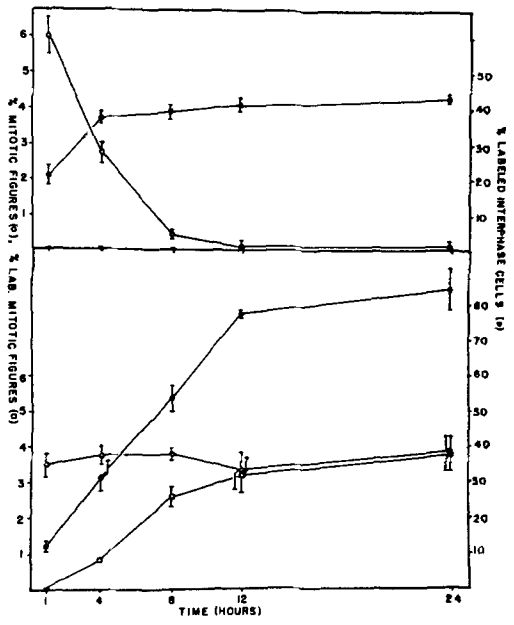

Fig. 5. Composite graph to show all cell cycle progression events for a control (bottom panel) and 2.8×10^{-6} M chlorsulfuron treatment (top panel). The G_1 to S progression is shown by (closed circles), the G_2 to M progression by (open circles), and the S to G_1 to M progression by (open squares).

In the chlorsulfuron treatment, progression of cells into S is inhibited after 4 h, indicating a G_2 block. The slope of entry during the first 4 h was the same as the control, but the number of cells proceeding into S was reduced about 50%.

With 2.8×10^{-6} M chlorsulfuron, a gradual entry inhibition of mitotic figures from G_2 into mitosis occurs (Fig. 5—top panel). With this treatment, labeled mitotic figures do not occur, indicating that no cells previously in S were able to pass through G_2 into M in the presence of chlorsulfuron.

Stationary Phase Experiment

In this experiment, 1-cm root tips were excised and placed for 72 h into sterile White's medium (White 1943) lacking sucrose. Under such conditions, meristematic cells will stop cycling and will become arrested in G_1 and in G_2 with no cells in S or M (Van't Hof 1968). This accumulation response, called the stationary phase, is reversible by transferring roots into medium containing sucrose. If radioactive thymidine is also added to the medium, it is possible to monitor the progession of G_1 cells into S and G_2 cells into mitosis.

The data in Fig. 6 represents a stationary phase experiment using chlorsulfuron at 2.8×10^{-6} M. The percent labeled interphase curve for the control shows that after a delay of almost 8 h cells previously arrested in G_1 progress into S (Fig. 6B). After 24 h approximately 25% of the interphase cells are labeled. In the treatment, slightly over 1% of the interphase cells were labeled after 24 h, indicating a significant but incomplete G_1 block (Fig. 6A). The percent mitotic figure curve in the control showed a lag of 4–8 h before the

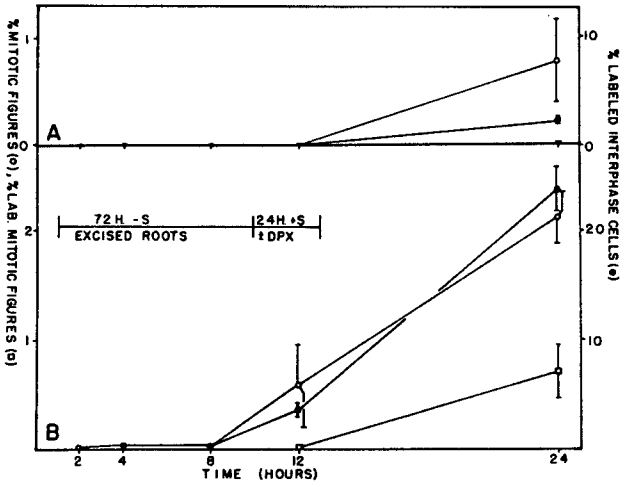

Fig. 6. Progression of cells previously arrested in G_1 and G_2 cells in a control (bottom panel) and 2.8×10^{-6} M chlorsulfuron treatment. The symbols are as follows: G_1 to S (open circles), G_2 to M (closed circles), and G_1 to S to M (open squares).

previously arrested G_2 cells entered mitosis (Fig. 6B). The number of mitotic figures increased to more than 2% after 24 h. After chlorsulfuron treatment this number was also significantly, but not completely, eliminated, indicating a partial block of previously arrested G_2 cells (Fig. 6A). The last measurement was of labeled mitotic figures. This curve measures the progression of cells previously arrested in G_1 after they transit S and G_2 and then divide. In the control, after a lag of 12 h, 0.5% labeled mitotic figures were observed (Fig. 6B). After chlorsulfuron treatment no labeled mitotic figures appeared in any sample. This result indicated that those cells previously arrested in G_1 which progressed through S in the presence of chlorsulfuron were not able to transit G_2. These observations reinforce the hypothesis that chlorsulfuron blocks G_2 and also suggests a secondary block in G_1. It also may indicate that chlorsulfuron acts especially on an event occurring in early G_2, since some of the arrested G_2 cells were able to divide, but none of the cells previously arrested in G_1 appeared as labeled mitotic figures.

Cell Cycle Distribution

The distribution of cells in the stages of the cell cycle was measured microspectrophotometrically. One hundred nuclei were measured in four roots (Fig. 7). Cells in prophase were used as an internal standard to estimate the range of G_2 values.

Panel A indicates the distribution of cells in the cell cycle in terms of relative DNA units. The arrowhead indicates the average value for G_2. Panel B shows the redistribution of these cells after 24 h of growth in the presence of 2.8×10^{-6} M chlorsulfuron. The relative number of cells in the G_2 portion of the cell cycle is increased. In panel C, cells in different stages of the cell cycle are indicated after 24 h in 2.8×10^{-5} M chlorsulfuron. In this case the percentage

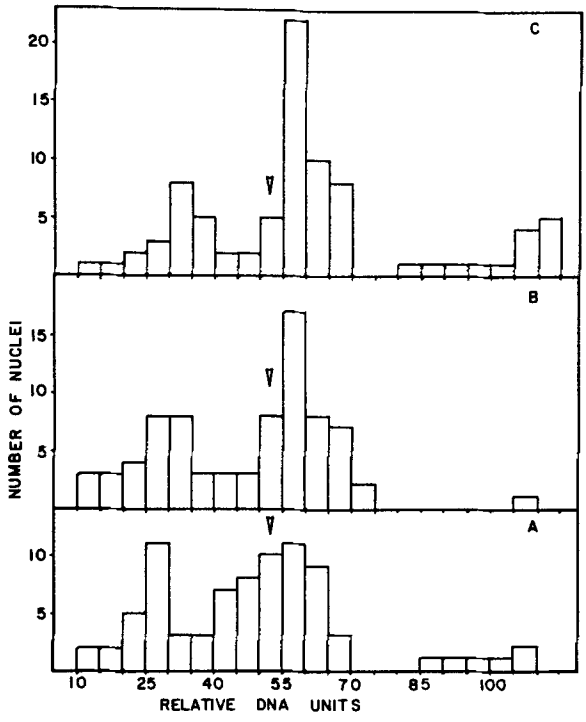

Fig. 7. The distribution of cells in the cell cycle in 2-mm root tip meristems based upon relative DNA unit amounts. The arrow heads indicate the average G_2 DNA value as determined by measuring the DNA amount of cells in prophase. A is the control, B is after 24 h of 2.8×10^{-6} M chlorsulfuron treatment, and C is after treatment at 2.8×10^{-5} M.

of cells in G_2 apparently has increased, reinforcing our interpretation that the transit of cells from G_2 into mitosis is being inhibited, thereby causing an increase in the size of this population.

RNA and Protein Synthesis

Roots of intact seedlings were immersed in Hoagland's solution with and without 2.8×10^{-6} M chlorsulfuron plus ^{3}H-uridine. Incorporation of uridine, used to estimate RNA synthesis, was reduced by 43% after 24 h of chlorsulfuron treatment. A deviation from the control was apparent from 1 h and was increased continuously over 12 h. After 12 h the degree of inhibition remained constant (Fig. 8A).

Protein synthesis was measured by incorporation of ^{3}H-protein hydrolysate into roots over a 24-h period with and without 2.8×10^{-6} M chlorsulfuron (Fig. 8B). A slight reduction in incorporation was observed between 8 and 12 h of chlorsulfuron treatment, but no difference was observed after 24 h. Chlorsulfuron partially inhibits RNA synthesis, but apparently not protein synthesis.

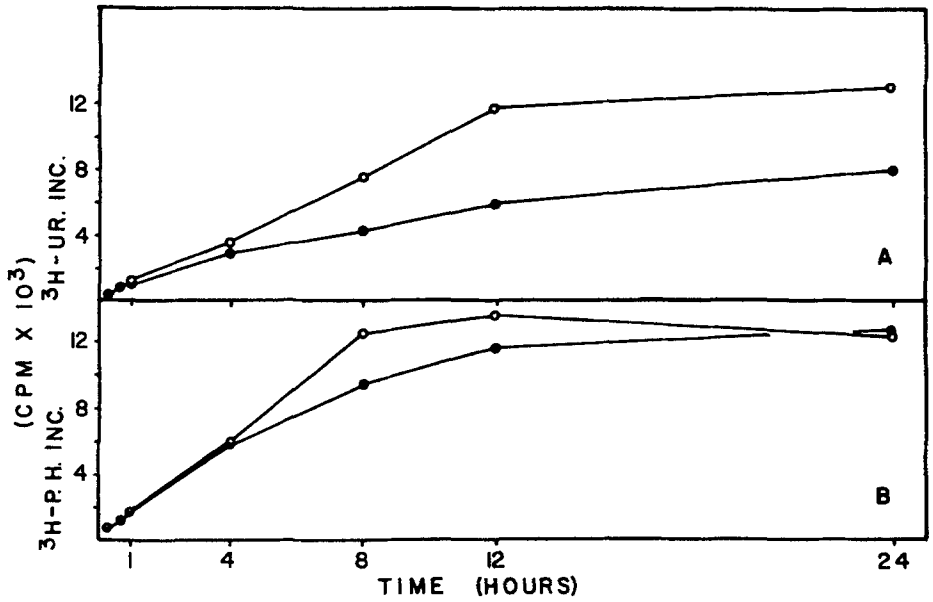

Fig. 8. Data showing incorporation data for tritiated uridine into RNA (A) and ^{3}H-protein hydrolysate into protein (B) in a control (open circles) and 2.8×10^{-6} M chlorsulfuron treatment (closed circles).

Discussion

In a cycling cell, RNA synthesis occurs in a particular pattern and sequence relative to the stages of the cell cycle. For example, in cultured Jerusalem artichoke tubers a step increase in RNA synthesis occurs during G_1 and G_2 (Mitchell 1969). In pea roots cultured in minus sucrose medium for 48 to 72 h, all cells stop cycling and are arrested in G_1 and G_2 (Van't Hof 1968). This happens because sucrose starvation has shut down macromolecular synthesis necessary for cycle progression. Webster and Van't Hof (1970) demonstrated that the specific inhibition of macromolecular synthesis inhibits cell cycle progression in previously starved root tips. RNA synthesis was not a clear response, however, because some long-lived mRNA is apparently present in starved pea root cells. This mRNA must be depleted before a specific RNA synthesis inhibitor could show its effect.

Certain specific proteins must be coded and synthesized before the G_1 to S and the G_2 to M transition can occur (Webster and Van't Hof 1970). In addition, certain initial amounts and species of RNA are most likely required to support cell cycle related metabolic events.

Chlorsulfuron experiments have been conducted on a variety of plants and experimental protocols. DeVilliers et al. (1980) used isolated bean cells to measure photosynthesis, respiration, and RNA, protein, and lipid synthesis. They reported basically no effects whatsoever at concentrations lower than

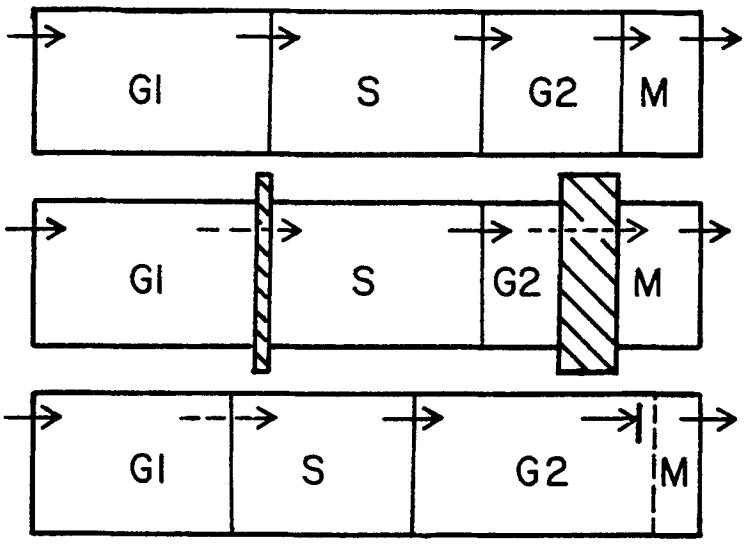

Fig. 9. Diagram showing the relative distribution of cells in a proliferating meristem (top), the position of cell cycle blocks induced by chlorsulfuron (middle), and the redistribution of cells after inhibitor treatment (bottom).

10^{-5} M. At 5×10^{-4} M, photosynthesis was inhibited 91% after a 2-h treatment, RNA synthesis was inhibited 67%, protein synthesis 59%, and lipid synthesis 65%. Bean cells are obviously quite resistant to chlorsulfuron. DeVilliers et al. suggest that photosynthesis is probably not the primary mode of action of chlorsulfuron.

The observations of Ray (1982) were presented in the introduction. Using a large number of assay systems, he concluded that chlorsulfuron did not inhibit respiration, photosynthesis, or protein synthesis. Hormone-induced cell enlargement was unaffected, but overall plant growth in corn was reduced within 2 h. In *Vicia faba* roots the number of mitotic figures was reduced by 87% over an unreported treatment duration, and in corn roots chlorsulfuron showed an 80% reduction in DNA synthesis after a 6-h treatment. From these results, Ray concluded that chlorsulfuron inhibits plant growth by inhibition of cell division at some stage prior to division.

DeVilliers et al. (1980) reported a 67% inhibition of RNA synthesis in isolated bean leaf cells with 5×10^{-4} M chlorsulfuron after a 2-h treatment. Ray (1982) showed a 28% RNA synthesis reduction in intact corn roots and no reduction in protein synthesis after a 6-h treatment. In my study on pea roots, a 43% reduction in RNA synthesis, as measured by ^{3}H-uridine incorporation, was observed over a 24-h treatment duration with 2.8×10^{-6} M chlorsulfuron. Only a small reduction in protein synthesis was observed.

Even though the three sets of experimental evidence are on different systems and were done using different methods, it is possible to draw a similar conclusion. Chlorsulfuron does not significantly inhibit protein synthesis, but it does have a partial inhibitory effect on RNA synthesis. Since specific species of

RNA are required for cell cycle progression, it is entirely feasible that chlorsulfuron inhibits the synthesis of cell cycle specific RNAs. This inhibition in turn would preclude the synthesis of cell cycle specific proteins and enzymes without causing an overall significant reduction in protein synthesis. The obvious next step to verify this hypothesis would be to determine the nature of RNA populations before and after chlorsulfuron treatment.

This study is a clear demonstration of the principal control point hypothesis (Van't Hof and Kovacs 1972). This hypothesis states that in order for cell cycle progression to occur, certain biochemical events must first be accomplished in G_1 and G_2. If these events are blocked in any way, synthesis of DNA or mitosis cannot proceed.

The experiments discussed show that chlorsulfuron at the three concentrations tested inhibit the progression of G_2 cells into mitosis. This was demonstrated by the inhibition of the labeled interphase curve after 4 h (Figs. 1, 2, and 5) and by the inhibition of the progression of cells into mitosis soon after chlorsulfuron treatment (Figs. 3, 4, and 5). The primary block to cell cycle progression appears to be in G_2 (Fig. 9).

A secondary block is also suggested in G_1 (Fig. 9). The evidence for this is based on rapid, though incomplete, reduction in ^{3}H-thymidine incorporation into root cells (Fig. 2) and by the reduction in percent labeled interphase cells after 4 h of treatment with 2.8×10^{-5} M and 2.8×10^{-4} M chlorsulfuron (Fig. 1). This last evidence could also be interpreted as a G_2 inhibition response. The stationary phase experiment (Fig. 6) provides clearer evidence of a G_1 block. In that case, the significant reduction in percent labeled interphase cells can only mean that cells previously arrested in G_1 cannot progress into S.

These positional blocks could be caused by cell cycle specific RNA synthesis inhibition. Continuous treatment with chlorsulfuron over the short term would cause a cell cycle redistribution (Fig. 9) with a greater number of cells in G_2. Treatment over the long term, more than 24 h, would deplete the meristem of cycling cells and could ultimately cause organ death.

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